The cytotoxicity and synergistic potential of aspirin and aspirin analogues towards oesophageal and colorectal cancer

[1]Kilari, R.S., [1,2]Bashir, A.I.J., [1]Perry, C.J., [3]Safrany, S.T., and [1]Iain D. Nicholl

[1]Research Institute in Healthcare Science, University of Wolverhampton, Wolverhampton WV1 1 LY, UK

[2]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Gombe State University, Gombe-Nigeria

[3]RCSI Bahrain, P.O. Box 15503, Adliya, Kingdom of Bahrain

Correspondence to: Dr Iain D. Nicholl, Department of Biomedical Science and Physiology, Faculty of Science and Engineering, University of Wolverhampton, Wolverhampton, WV1 1LY, UK

Email: I.Nicholl@wlv.ac.uk

[1]ORCID No. 0000-0003-3803-7039

Abbreviations: ADC, adenocarcinoma; BO, Barrett's oesophagus; CRC, colorectal cancer; ED, effective dose; IC, inhibitory concentration; OC, oesophageal cancer; SA, salicylic acid; SSC, squamous cell carcinoma

Key words: oesophageal cancer, platins, aspirin, aspirin analogues, apoptosis

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Abstract: ***Background:*** Oesophageal cancer (OC) is a deadly cancer because of its aggressive nature with survival rates that have barely improved in decades. Epidemiologic studies have shown that low-dose daily intake of aspirin can decrease the incidence of OC.

Methods: The toxicity of aspirin and aspirin derivatives to OC and a CRC cell line were investigated in the presence and absence of platins.

Results: The data in this study show the effects of a number of aspirin analogues and aspirin on OC cell lines that originally presented as squamous cell carcinoma (SSC) and adenocarcinoma (ADC). The aspirin analogues fumaryldiaspirin (PN517) and the benzoylsalicylates (PN524, PN528 and PN529), were observed to be more toxic against the OC cell lines than aspirin. Both quantitative and qualitative apoptosis experiments reveal that these compounds largely induce apoptosis, although some necrosis was evident with PN528 and PN529. Failure to recover following the treatment with these analogues emphasized that these drugs are largely cytotoxic in nature. The OE21 (SSC) and OE33 (ADC) cell lines were more sensitive to the aspirin analogues compared to the Flo-1 cell line (ADC). A non-cancerous oesophageal primary cells NOK2101, was used to determine the specificity of the aspirin analogues and cytotoxicity assays revealed that analogues PN528 and PN529 were selectively toxic to cancer cell lines, whereas PN508, PN517 and PN524 also induced cell death in NOK2101. In combination index testing synergistic interactions of the most promising compounds, including aspirin, with cisplatin, oxaliplatin and carboplatin against the OE33 cell line and the SW480 colorectal cancer (CRC) cell line were investigated. Compounds PN517 and PN524, and to a lesser extent PN528, synergised with cisplatin against OE33 cells. Cisplatin and oxaliplatin synergised with aspirin and PN517 when tested against the SW480 cell line.

Conclusion: These findings indicate the potential and limitations of aspirin and aspirin analogues as chemotherapeutic agents against OC and CRC when combined with platins.

1. INTRODUCTION

]Worldwide, oesophageal cancer (OC) is estimated to be the 7th most commonly diagnosed cancer, with over 570,000 developing the disease and approximately 500,000 deaths predicted to occur in 2018 [1]. A significant proportion of patients present with advanced or metastatic disease upon diagnosis [2]. More than half of OC diagnosed is an adenocarcinoma (ADC), originating from the distal third of the oesophagus and is associated with gastric reflux and Barrett's oesophagus (BO), where columnar epithelium replaces the normal squamous epithelium [3]. By contrast, squamous cell carcinoma (SCC) largely arises above the tracheal bifurcation and is responsible for more than a quarter of cases of OC [4]. Given that OC overall has a dismal 5-year survival rate of 19.2% (USA: https://seer.cancer.gov/statfacts/html/esoph.html) and with survival rates barely improving in over a generation, there is a need to identify novel chemotherapeutic agents that have the potential to target this cancer and/or can synergise with current treatment modalities. This need is underscored by the finding that the incidence of oesophageal adenocarcinoma appears to be on the rise [5].

Several studies have reported that regular intake of aspirin (acetylsalicylic acid) or non-steroidal anti-inflammatory drugs (NSAID) can result in reduced morbidity or mortality from gastrointestinal cancer. For example, Thun *et al.* suggested that death rates decreased with aspirin usage for colorectal, stomach and oesophageal cancer in comparison to other cancers [6]. Funkhouser and Sharp, and Farrow *et al.* found that aspirin users were at a significantly decreased risk from OC [7, 8]. In addition, in a meta-analysis study based on exposure type, aspirin usage was found to have greater protective effect than non-aspirin NSAIDs against the development of OC [9], but any use was protective against both histological types (ADC: OR=0.67 and SCC: OR=0.58]. Two more recent meta-analyses lend credence to the protective nature of regular aspirin use: Bosetti *et al.* estimates a statistically significant reduced relative risk of 0.64 for squamous cell oesophageal cancer [10], and Cuzick *et al.* report a best estimate risk ratio of 0.7 for incidence and 0.5 for mortality for OC [11]. Barrett's oesophagus patients also using NSAIDs exhibit a significantly reduced risk of developing oesophageal ADC (6.6%) compared to non-users (14.3%) [12].

It has been suggested that the reduced cancer risk in BO patients using NSAIDs could be a consequence of a decreased rate of acquisition of somatic genomic abnormalities [13], an intriguing finding given that inflammation and genetic instability is intimately linked (e.g. see [14]. NSAIDs can also prevent OC experimentally; for example, indomethacin reduced diethylnitrosamine induced OC in mice [15] and ursodeoxycholic acid in combination with aspirin reduced the risk of oesophageal ADC in a rat model of BO [16]. Studies suggest that aspirin (and indeed other NSAIDs) exert their anti-cancer effects through cyclooxygenase (COX)-dependent and COX-independent pathways [17-20]. Aspirin inhibits PG production by the irreversible acetylation and inhibition of the cyclooxygenase activity in PGHS [21]. Overexpression of COX-2 in tissues has been intimately linked with cancer progression [22]. Prostaglandin E2 (PGE2), a major prostanoid produced by COX-2, promotes cell proliferation, metastasis, angiogenesis and resistance to apoptosis [23]. Increased COX-2 expression is seen in BO and human oesophageal cancer (SCC and ADC) [24-27]. The evidence for a role of COX1 in the pathophysiology of cancer is weaker [28, 29].

The evidence supporting COX-independent targets for aspirin (and the metabolite salicylate) is increasing, and include (but are not limited to) the NF-κB pathway [30, 31] which is critically important in

inflammatory responses, activation of the p38 MAP kinase pathway with subsequent cyclin D1 degradation [32], down-regulation of *Bcl-2* expression [33] and mTOR inhibition and activation of AMP-activated protein kinase [34, 35]. Aspirin usage *after* a diagnosis of colon cancer also has a positive outcome: longer survival is noted among patients with mutated-*PIK3CA* colorectal cancer, but not with wild type *PIK3CA* cancer [36]. Taken together, these observations suggest that aspirin can act pleiotropically. We have identified aspirin analogues that are more potent than aspirin with respect to inhibition of colorectal cancer cell line growth *in vitro* and *in vivo*, have the capacity to induce apoptosis, inhibit NF-κB and reduce cyclin D1 levels [37, 38]. Herein, we have extended our investigation into aspirin analogues (Table 1) and examined their cytotoxicity to the OC cell lines OE21, OE33 and Flo-1, given the evidence that aspirin may be chemoprotective against OC.

2. MATERIALS and METHODS

2.1 Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, foetal bovine serum (FBS), L-glutamine and antibiotic solution were from Life Technologies Ltd or Gibco (Paisley, UK). Epithelial cell medium (EpiCM) kit was from ScienCell research laboratories (Carlsbad, CA, USA). The Annexin-V-Fluos staining kit for flow cytometry was from Roche Diagnostics Operations Inc. (Indianapolis, USA). Carboplatin, oxaliplatin and cisplatin (cis-diamminedichloroplatinum (II)) were from Stratech Scientific Ltd., Newmarket, UK. Dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other reagents, unless otherwise specified were obtained from Sigma-Aldrich Company Ltd (Dorset, UK).

2.2 Compound synthesis.

Preparation of PN510. This was prepared in an analogous way to PN508 [37] using adipoyl chloride in lieu of succinyl chloride. The off-white solid had a melting range of 174 -176^{o}C [lit.,171-174^{o}C [39]].

Preparation of PN526 and PN527. These carbonate esters were prepared by a variation of the general method for synthesis of alkanoylsalicylate analogues according to Deb *et al*. [37], but using methyl chloroformate and ethylchloroformate, instead of an acid chloride, respectively. As an example, PN527 was synthesized by placing salicylic acid (6.9 g; 0.05 mol) to a 250 ml round bottomed flask fitted with a clamp, condenser, a small dropping funnel and magnetic stirrer. Diethyl ether (dried over anhydrous magnesium sulphate, 24 h, 100 ml) was added and stirred until the salicylic had completely dissolved, at which point pyridine (4 ml; 0.05 mol) was added. The apparatus was fitted with calcium chloride guard tubes, throughout, to exclude moisture. Ethyl chloroformate (5.47 ml; 0.05 mol) was added through the dropping funnel (drop wise) over a period of 10 min. The mixture was then vigorously stirred for a further 30 min, after which a precipitate of pyridinium chloride was clearly visible. A further 50 ml dry diethyl ether was added and the solution was stirred for 10 min. The solution was filtered into a 500 ml conical flask and deionized water (100 ml) was added. The solution was vigorously stirred for 5 min before the ether layer was separated, dried over anhydrous magnesium sulphate and rotary-evaporated to dryness. The crude product (9.1 g; 93%) was an oil that soon crystallised. The product was dissolved in hot toluene (25-30 ml), cooled and stored at -15°C for 1 week to give a colourless solid: (yield 7.20

g); IR: ν C=O acid 1682/cm^{-1}, ν C=O ester (1) 1756/cm^{-1}, ν C=O ester (2) 1756/cm^{-1}; mp: 96-98˚C (lit., not found).

For PN526; crude yield = 79% recrystallised from ethanol in a similar manner to that described above; IR: ν C=O acid 1682/cm^{-1}, ν C=O ester (1) 1757/cm^{-1}, ν C=O ester (2)1757/cm^{-1}; mp: 140-142˚C (lit. 134˚C, U.S. Environmental Protection Agency: https://comptox.epa.gov/dashboard/dsstoxdb/results?search=Carbonic+acid,+methyl+ester,+ester+with+salicylic+acid).

2.3 Compound preparation. Aspirin and aspirin analogues were prepared as fresh stock solutions in DMSO, usually at a concentration of 0.25 M, before each experiment.

2.4 Cell culture. The human oesophageal cancer cell lines and primary oral keratinocyte NOK2101cell line were obtained from Mr Tim Underwood (University of Southampton). The OC cell lines OE21 (of SCC origin) and OE33 (of ADC origin) [40] were cultured in RPMI-1640 medium with L-glutamine supplemented with 10% (v/v) heat inactivated FBS and penicillin-streptomycin. The Flo-1 OC cell line (of ADC origin) [41] was cultured in DMEM medium with L-glutamine supplemented with 10% heat inactivated FBS and penicillin-streptomycin solution. The primary keratinocyte cells, NOK2101 were maintained in EpiCM medium supplemented with 10% FBS and penicillin-streptomycin solution. The SW480 colon adenocarcinoma cell line (ECACC, Salisbury, UK) was cultured in Leibovitz L-15 medium (Thermo Fisher Scientific) containing 10% (v/v) FBS supplemented with L-glutamine-penicillin-streptomycin in sealed culture flasks. The cells were cultured at 37ºC in a humidified incubator with 5% CO_2 and regularly passaged at ~80% confluency.

*2.5 Anti-proliferative potency of compounds***.** The cytotoxic effect of aspirin analogues on the oesophageal cancer cells and the non-cancerous keratinocyte cell line was tested using the MTT assay [42]. Briefly, 2.5 ×10^3 (OE21 cells) or 10^4 cells/well were seeded in 96-well microtitre plate and cultured overnight. After 24 h of initial seeding, the culture medium was replaced with fresh medium containing drugs or vehicle control and incubated at 37ºC. On completion of incubation, the supernatant was aspirated and cells incubated with 300 µl of MTT substrate (0.5 mg/ml) for 3 h. The supernatant was removed and the resultant formazan crystals were dissolved in 200 µl of DMSO. The absorbance was read at 540 nm using a microplate reader (Microplate Reader Thermo Multiskan Ascent 96 & 384). Metabolic activity (viability) is reported as the percentage of treated cells relative to the untreated cells in control wells. Both readings were background subtracted, using the absorbance at time zero. Growth inhibition values (IC_{50}) were determined using a non-linear sigmoidal plot with 4 variable parameters (Minimum, maximum, IC_{50} value and Hill slope were not fixed). pIC_{50} (-logIC_{50}), minimum, maximum and Hill slopes were treated as being normally distributed, allowing parametric analyses to be performed. All data analysis was performed using GraphPad Prism Statistics Software package (ver. 6.0; San Diego, CA, USA).

2.6 Apoptosis with Annexin-V-fluos staining and flow cytometry analyses. The ability of aspirin analogues to induce apoptosis was tested *in vitro* using a commercially available Annexin-V-Fluos cell staining kit. Briefly, cells were cultured in 25 cm^2 flask until they reached ~50% confluency. Cells were incubated for the indicated

time with compounds (1 mM). Following incubation, cells were harvested and washed in Hank's balanced salt solution (HBSS) buffer and incubated with labelling solution (100 µl incubation buffer with 2 µl Annexin-V-Fluos reagent and 2 µl of propidium iodide (PI) used for ~10^6 cells) for 10-15 min at room temperature as per the manufacturer's instructions. Cells were further diluted, if required, with HBSS buffer and analysed on a flow cytometer (BD Biosciences, Facscalibur). A positive control for apoptosis (irinotecan, 25 µM), positive control for necrosis (H_2O_2, 2 mM) and untreated control cells were included in the experiment for comparison. Flow cytometry settings used were excitation wavelength of 488 nm and bandpass filter of 530 nm for fluorescein (green) detection and for PI, a bandpass filter of 600 nm was employed. Use of the PI counter-stain with the principal stain enabled discrimination of viable cells from non-viable cells and apoptotic cells from necrotic cells. Viable cells do not take up either stain whereas non-viable cells take up only PI. Apoptotic cells take up the Annexin-V/ApoTRACE stain but not PI whereas necrotic/late apoptotic cells take up both principal and PI stain. For detection of early apoptosis and late apoptosis/necrosis, cells were gated into three populations: Annexin-/PI- (viable), Annexin+/PI- (early apoptotic), and Annexin+/PI+ (late apoptotic/necrotic).

*2.7 Evaluation of cytotoxicity and cytostaticity of compounds***.** OE21, OE33 and Flo-1 cells were seeded at a density of 5×10^3, 2×10^4 and, 2×10^4 cells/well respectively, in 96-well plates in their respective basal medium. After 24 h seeding, the cells were treated with compound-containing culture medium for a further 72 h; cells were treated with either aspirin compounds (1 mM), irinotecan (25 µM), H_2O_2 (2 mM) or vehicle control. The supernatant was removed and adherent cells were harvested and washed with phosphate buffered saline. Cells were then reseeded, at the same density as the initial seeding, in a fresh plate with drug-free medium and allowed to recover for 72 h at 37ºC. Following this recovery period, cells were collected by trypsinization and counted using a Beckman Coulter Z1 cell counter with an orifice of 100 µm and threshold size of 10 µm.

2.8 Synergy with conventional anticancer drugs. OE33 cells were plated in a 96-well plate at a density of 1.0 X 10^4 cells/well and incubated overnight at 37°C. The cells were then treated with serially diluted concentrations of cisplatin, oxaliplatin, carboplatin, aspirin and its analogues for 72 h [43-47]. Cell viability was measured by MTT assay and the IC_{50} was calculated for each compound. Combination drug treatments were then performed as above by pairing cisplatin, oxaliplatin or carboplatin with each of the aspirin analogues in a constant ratio design based on the IC_{50}. All assays were performed in duplicates (N=3). CompuSyn software ver. 1.0 published and distributed by ComboSyn Inc., (Paramus, NJ., USA) was used to calculate the Combination Index (CI) and Dose Reduction Index (DRI). This produces multiple drug dose-effect calculations using the Median Effect methods described by Chou and Talalay [48]. The CI is the quantitative measure of the degree of drug interaction in terms of additive effect (CI = 1), synergism (CI < 1), or antagonism (CI > 1) while the DRI is the measure of favourable dose reduction when two drugs are used in combination. Graphpad Prism 7 software (ver. 7.0a, April 2016) was used to calculate the IC_{50} of individual compounds and their respective combinations. A description and interpretation of synergism or antagonism in combination studies using the CI method of analysis [adapted from [49, 50]] is presented (Table 2).

3. RESULTS

3.1 Aspirin analogues show anti-proliferative activity against oesophageal cancer cells.

Using the MTT cell viability assay a number of aspirin analogues including 'diaspirins': PN508, PN510, PN511, PN512 and PN517; 'benzoylsalicylates': PN514, PN524, PN525, PN528 and PN529; and 'carbonates', PN526 and PN527, were screened for their efficacy to kill oesophageal cancer cells (Figure 1). Aspirin and salicylic acid (SA) were included in the experiment as controls. Assay results demonstrated that the benzoylsalicylates; PN524, PN528 and PN529 to be substantially more potent in killing OE21 and OE33 cells than PN508 and aspirin itself (Figures 1A, 1B). PN517 was not as effective as the benzoylsalicylates, exhibiting modest cytotoxicity to these cells. Conversely, the Flo-1 cell line was resistant to most of the compounds tested other than PN528 and PN529 (Figure 1C). PN514 was more potent in killing OE21 cells, whereas PN512 was more cytotoxic to OE33 cells. Other compounds including PN510, PN511, PN525, PN526 and PN527 failed to show robust cytotoxicity towards the three cancer cell lines (Figure 1) and were not investigated further. MTT analysis of aspirin analogues on the non-cancerous primary cell type NOK2101 showed a rather complex and differential response compared to cancer cells. Aspirin and SA showed little or no killing of NOK2101 cells at the concentration tested. The differential toxicities of PN528 and PN529 is noteworthy. They were most effective against the oesophageal adenocarcinoma OE33 and Flo-1, causing over 75% reduction in the MTT assay. In contrast, they only reduced NOK2101 proliferation by under 35% and of the analogues, PN512 and PN514 showed the least cytotoxicity to the NOK2101 cells.

Using MTT data, dose response curves were determined and IC_{50} values were calculated for each of the aspirin analogues (Table 1). The compounds were found to suppress the proliferation of OC cells in a dose-dependent manner (data not shown). PN529 was the most cytotoxic compound, with IC_{50} values in the range of 100-400 μM. In comparison, aspirin ranged between 1 mM and 7 mM. Determination of the IC_{50} values indicates OE21 cells are more sensitive to these compounds.

3.2 Aspirin analogue-mediated cancer cell killing is through induction of apoptosis. To examine whether treatment with aspirin analogues resulted in cell death through apoptosis or necrosis, treated cells were stained and subsequently imaged using fluorescence microscopy. Controls for apoptosis (irinotecan) and necrosis (H_2O_2) were also used. The quantitative assay utilized staining of apoptotic cells with Annexin-V, which shows affinity to phosphatidylserine on the cell surface, with analysis using flow cytometry [51]. Following 48 h treatments, analysis suggests that most of the analogues exert their toxicity through the induction of apoptosis (Figure 2). However, some necrosis was observed upon incubation with PN528 and PN529 (data not shown). Flow cytometric analysis at 72 h revealed PN524 and PN517 to be the leading analogues inducing apoptosis in OE21 and OE33 cells; in contrast PN528 and PN529 were found to induce more apoptosis in Flo-1 cells (Figure 2).

3.3 Aspirin analogues are cytotoxic in nature. A cell recovery assay was performed to examine whether treatment with aspirin and aspirin analogues induced cytostasis or cell death. In general, treatment with irinotecan or H_2O_2 was detrimental to the cancer cells at the concentrations tested with cells failing to recover during the recovery period. OE21 cells failed to thrive following treatment with compounds (Figure 3). In contrast OE33 cells treated with aspirin appear to grow more rapidly upon its removal. This trend may also be

present in Flo-1 cells. In contrast, PN528 and PN529 showed cytotoxic effects towards all OC cells tested. PN517 and PN524 appeared to be more cytotoxic to OE21 cells and cytostatic towards Flo-1 cells.

3.4 Combination index testing of platins and salicylates in OE33 and SW480 cancer cells. We aimed to establish what effect combining aspirin and aspirin analogues with platins would have against the OE33 (chosen given the intermediary nature of these cells' responses to compounds) and the SW480 colorectal cancer cell line. Identifying a means to decrease the dose needed for these platinum compounds is of importance in clinical care because of their narrow therapeutic index [52]. Given the findings reported herein regarding toxicity, specificity and apoptotic and necrotic potential, we investigated the capacity of platins to synergise with selected compounds including aspirin (PN502), PN517, PN524 and PN528.

Cisplatin in combination with aspirin [1:100 ratio] at the ED_{50} against the OE33 oesophageal cancer cells had an antagonistic effect. Synergistic effects were observed however, when cisplatin was combined with PN517 [1:50] with the ED_{50} for cisplatin reduced from 5.4 μM to 1.5 μM. Synergistic effects at ED_{50}, ED_{75} and ED_{90} were also observed when cisplatin was combined with PN524 [1:50] against the oesophageal cell line and was accompanied by a reduction in the ED_{50} of cisplatin from 5.4 μM to 2.3 μM. The ED_{50} for cisplatin when used in combination with PN517 [1:50] was reduced from 5.4 μM to 1.5 μM while that of PN517 was reduced from 600 μM to 75 μM. The ED_{50} was reduced from 5.4 μM and 408 μM to 2.3 μM and 113 μM respectively for cisplatin and PN524 [1:50] in combination. The cisplatin and PN528 combination resulted in a decrease in ED_{50} for cisplatin from 5.4 μM to 3.5 μM. Oxaliplatin in combination with aspirin [1:20] had synergistic effects at ED_{50} and below, which indicates synergy at low doses with little cytotoxic effects, but this is not relevant in cancer therapy. Oxaliplatin in combination with PN517 and PN524 against the OE33 oesophageal cancer cell line had antagonistic effects at ED_{75} and ED_{90} with synergy at ED_{50} and below. Antagonistic effects were also observed when carboplatin was used in combination with aspirin and PN517, but in combination with PN524 [1:20], however, had a synergistic effect at ED_{50} and below with combinations having antagonistic effects at ED_{75}, and ED_{90}. The ED_{50} of carboplatin when combined with PN524 was reduced from 40.8 μM to 11.7 μM, which is an approximately 4-fold reduction in dose to kill 50% of the oesophageal cancer cells. PN524 in combination with oxaliplatin and carboplatin had synergistic effects at ED_{50} and lower. Antagonism was noted when oxalipatin and carboplatin were tested in combination with PN528, although slight/moderate synergy was observed with cisplatin at ED_{75} and ED_{90} respectively.

In contrast, in the SW480 CRC cell line, synergy/strong synergy was observed when aspirin was combined with cisplatin and oxaliplatin. Moreover, synergy was observed when cisplatin was combined with PN517 [1:10] with a 5-fold decrease in the ED_{50} for cisplatin. Remarkably, the ED_{50} for oxaliplatin had a 7-fold decrease when combined with PN517 resulting in a strong synergistic effect which was also apparent at ED_{75} and ED_{90}. Cisplatin in combination with PN524 [1:40] had synergistic effects with about a 7-fold decrease in ED_{50} for cisplatin. The synergistic effects of these combinations increased at higher dose, which is advantageous in chemotherapy (data not shown). However, although there was a synergistic effect when oxaliplatin was combined with PN524 [1:8] at ED_{50}, the effect regressed to antagonism with increased dose (ED_{75} and ED_{90}), a result that is not likely to produce the maximum cytotoxicity required in cancer therapy [53]. Moreover, the dose of carboplatin at ED_{50} increased rather than decreased when used in combination with the aspirin analogue PN524 (101 μM alone to 152 μM in combination); this increase in ED_{50} of defeats one of the main reasons of

combination therapy, which is to achieve a decrease in effective dose in order to reduce or alleviate side effects. Some of the combinations showed strong antagonism due to very high CI values; this is possible in some combinations as the synergy scale is from 1 to 0 and the antagonism scale is from 1 to infinity [53]. The differences in outcomes for each drug combination in different cell lines may be due to differences in target specificity of the compounds [54].

For clarity, a summary of the effects of all the combinations as determined by their CI values against OE33 oesophageal and SW480 colorectal cancer cell lines calculated utilising CompuSyn can be found in Table 3.

4. DISCUSSION

Given the evidence that aspirin may be chemoprotective against OC, we have investigated the cytotoxicity of aspirin analogues to the OC cell lines OE21, OE33 and Flo-1. The aspirin analogues fumaryldiaspirin (PN517) and the benzoylsalicylates (PN524, PN528 and PN529), were observed to be more toxic against the OC cell lines than aspirin. Of note are PN528 and PN529, which were particularly effective against oesophageal adenocarcinoma cell lines, OE33 and Flo-1, while showing reduced toxicity towards non-cancerous NOK2101 cells. These compounds appear to act by inducing apoptosis. Some necrosis, albeit not statistically significant in our studies was evident with PN528 and PN529. This should not be attributed to late apoptosis as the same was not observed when irinotecan was used.

We obtained surprising results when looking at the ability of cells to recover following removal of the drugs. The "rebound effect" is well understood with aspirin when looking at cardiovascular effects [55]. An increase of cardiovascular events by 30% can be observed if low-dose aspirin is stopped. We are unaware of data looking at the incidence of cancer following such removal of treatment. Our observations with aspirin suggest tumours may grow more vigorously should treatment be halted. In contrast removal of PN528 and PN529 in particular showed they were largely cytotoxic and did not permit any rebound growth. The OE21 (SSC) and OE33 (ADC) cell lines were more sensitive to the aspirin analogues compared to the Flo-1 cell line (ADC). Utilising a non-cancerous oesophageal primary cell line NOK2101 revealed that analogues PN528 and PN529 had some selective toxicity to cancer cell lines, whereas PN508, PN517 and PN524 also induced cell death in NOK2101.

This issue of lack of specificity spurred us on to investigate whether synergistic action with platins commonly used in cancer treatment with the aspirin analogues and with aspirin itself was possible. In the OE33 oesophageal cancer cell line, none of the platinum compounds synergised with aspirin. Although there was synergy when oxaliplatin and aspirin were combined at ED_{50}, this effect regressed at ED_{75} and ED_{90} and thus is not favourable in the treatment of cancer. In gastric cancer cells however, cell growth was significantly inhibited when cisplatin was used in combination with aspirin [56]. In our studies, the combination of cisplatin and PN517 maintained its synergistic effects through ED_{50}, ED_{75} and ED_{90}, which make this a promising combination for the treatment of oesophageal cancer, as a synergistic effect between these two compounds is maintained at different dose effects. In contrast, oxaliplatin and carboplatin in combination with PN517 largely exhibited antagonistic effects; although there was moderate synergy between oxaliplatin and PN517 at ED_{50}, the

positive effect decreased as doses increased (at ED_{75} and ED_{90}), which is not favourable in the treatment of cancer because maximum cytotoxicity against the cancer cells is critical to effective therapy [53]. Cisplatin and PN524 exhibited synergy at ED_{50}, ED_{75} and ED_{90}. Although oxaliplatin and carboplatin showed synergistic effects with PN524 at ED_{50}, the effects declined at ED_{75} and ED_{90} respectively. Slight synergy was observed when cisplatin was tested in combination with PN528.

In the SW480 CRC cell line substantive synergy was observed when aspirin (PN502) and PN517 were combined with cisplatin and oxaliplatin with a decrease in ED_{50} for these platinum compounds. Surprisingly, antagonistic effects were observed when aspirin was used in combination with carboplatin. PN524 synergised with cisplatin. Given the common use of oxaliplatin in the treatment of colorectal cancer and with a clinically significant side effect of oxaliplatin being peripheral neuropathy [52], a reduction in its ED_{50} as a result of combination with aspirin and PN517 and PN524 may have clinical utility: a reduction in drug concentration will result in toxicity reduction and also delay or minimize the induction of drug resistance [53]. It is worth pointing out that whilst the concentration of the compounds tested in the experiments outlined may seem rather high in comparison to other chemotherapeutic agents, a range of 1-5 mM or above is not uncommon in *in vitro* and *in vivo* experiments investigating the molecular action of aspirin or salicylate [35, 54, 57, 58]

CONCLUSION

Notwithstanding the uncertainties regarding the precise molecular targets of aspirin and indeed, the aspirin analogues tested herein, we present evidence of synergistic potential with platinum compounds (particularly cisplatin) and suggest these findings should be extended to *in vivo* analyses in more clinically relevant models given the somewhat refractory nature of oesophageal and colorectal cancer. We find that the novel aspirin analogues were significantly more potent against oesophageal cancer cell lines than aspirin itself, and that the compounds can induce apoptosis. The inability of the cancer cells to recover following treatment with these analogues suggests that these drugs can be cytotoxic and not cytostatic in nature. The OE21 cell line (of SCC origin) was found to be the most sensitive cell line, whereas Flo-1 cells (ADC) were found to be more resistant. Perhaps most significantly, we also find that selected aspirin analogues can synergise with platins against the OE33 and SW480 cancer cell lines, and that aspirin can synergise with cisplatin and oxaliplatin against the SW480 cell line.

FIGURE LEGENDS

Figure 1. Screening aspirin analogues for their potency towards oesophageal cancer cell lines and a non-cancerous keratinocyte cell line *in vitro.* (A) OE21, (B) OE33, (C) Flo-1 and (D) NOK2101 cells were cultured for 24 h and the cells were then treated with compound-containing (1 mM) culture medium for 72 h. The anti-proliferative effects were then measured. Data are plotted as mean ± SEM (N=3). The effect of aspirin and salicylic acid (SA) is included for comparison. Two-tailed one-way ANOVA followed by Dunnett's *post hoc* test (comparing data with aspirin) showed * $p \leq 0.05$ and ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 2. Compound induced apoptosis in oesophageal cancer cell lines. Following 72 h treatment with compounds (1 mM), cells were stained with Annexin-V-Fluos and counter-stained with PI and the population of cells undergoing apoptosis was measured by FACS analysis. Irinotecan (IRI) and H_2O_2 were used as positive controls for apoptosis and necrosis, respectively. The effect of aspirin and DMSO (vehicle control) is included for comparison. Data plotted as mean ± SEM (N=3). Two-tailed one-way ANOVA followed by Dunnett's *post hoc* test (comparing data against vehicle control) showed * $p \leq 0.05$ and ** $p \leq 0.01$.

Figure 3. Recovery of oesophageal cancer cells following withdrawal of aspirin and aspirin compounds. OE21, OE33 and Flo-1 cells were treated with compound containing culture medium (1mM) for 72 h; viable/adherent cells were harvested, counted and re-seeded with compound-free-medium. After 72 h of recovery, cell counts were determined. The effect of aspirin, DMSO (vehicle control), irinotecan (IRI) and H_2O_2 is included for comparison. Data plotted as mean ± SEM (N=3, performed in duplicates). Two-tailed one-way ANOVA followed by Dunnett's *post hoc* test (comparing data against vehicle control) showed * $p \leq 0.05$ and ** $p \leq 0.01$.

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Competing interests

Dr CJ Perry and ID Nicholl are named inventors for a patent for F-DiA as an anti-colorectal cancer agent; US patent # 9,351,980.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018.
[2] Layke JC, Lopez PP. Esophageal cancer: a review and update. Am Fam Physician. 2006;73(12):2187-94.
[3] Reid BJ, Li X, Galipeau PC, Vaughan TL. Barrett's oesophagus and oesophageal adenocarcinoma: time for a new synthesis. Nat Rev Cancer. 2010;10(2):87-101.
[4] Siewert JR, Ott K. Are squamous and adenocarcinomas of the esophagus the same disease? Semin Radiat Oncol. 2007;17(1):38-44.
[5] Edgren G, Adami HO, Weiderpass E, Nyren O. A global assessment of the oesophageal adenocarcinoma epidemic. Gut. 2013;62(10):1406-14.
[6] Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath CW, Jr. Aspirin use and risk of fatal cancer. Cancer Res. 1993;53(6):1322-7.
[7] Farrow DC, Vaughan TL, Hansten PD, Stanford JL, Risch HA, Gammon MD, et al. Use of aspirin and other nonsteroidal anti-inflammatory drugs and risk of esophageal and gastric cancer. Cancer Epidemiol Biomarkers Prev. 1998;7(2):97-102.
[8] Funkhouser EM, Sharp GB. Aspirin and reduced risk of esophageal carcinoma. Cancer. 1995;76(7):1116-9.
[9] Corley DA, Kerlikowske K, Verma R, Buffler P. Protective association of aspirin/NSAIDs and esophageal cancer: a systematic review and meta-analysis. Gastroenterology. 2003;124(1):47-56.
[10] Bosetti C, Rosato V, Gallus S, Cuzick J, La Vecchia C. Aspirin and cancer risk: a quantitative review to 2011. Ann Oncol. 2012;23(6):1403-15.
[11] Cuzick J, Thorat MA, Bosetti C, Brown PH, Burn J, Cook NR, et al. Estimates of benefits and harms of prophylactic use of aspirin in the general population. Ann Oncol. 2015;26(1):47-57.
[12] Vaughan TL, Dong LM, Blount PL, Ayub K, Odze RD, Sanchez CA, et al. Non-steroidal anti-inflammatory drugs and risk of neoplastic progression in Barrett's oesophagus: a prospective study. Lancet Oncol. 2005;6(12):945-52.
[13] Kostadinov RL, Kuhner MK, Li X, Sanchez CA, Galipeau PC, Paulson TG, et al. NSAIDs modulate clonal evolution in Barrett's esophagus. PLoS Genet. 2013;9(6):e1003553.
[14] Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis. 2009;30(7):1073-81.
[15] Rubio CA. Antitumoral activity of indomethacin on experimental esophageal tumors. J Natl Cancer Inst. 1984;72(3):705-7.

[16] Rizvi S, Demars CJ, Comba A, Gainullin VG, Rizvi Z, Almada LL, et al. Combinatorial chemoprevention reveals a novel smoothened-independent role of GLI1 in esophageal carcinogenesis. Cancer Res. 2010;70(17):6787-96.
[17] Levy GN. Prostaglandin H synthases, nonsteroidal anti-inflammatory drugs, and colon cancer. FASEB J. 1997;11(4):234-47.
[18] Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J. 2001;15(12):2057-72.
[19] Elwood PC, Gallagher AM, Duthie GG, Mur LA, Morgan G. Aspirin, salicylates, and cancer. Lancet. 2009;373(9671):1301-9.
[20] Gurpinar E, Grizzle WE, Piazza GA. COX-Independent Mechanisms of Cancer Chemoprevention by Anti-Inflammatory Drugs. Front Oncol. 2013;3:181.
[21] Vane JR, Botting RM. The mechanism of action of aspirin. Thromb Res. 2003;110(5-6):255-8.
[22] Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene. 1999;18(55):7908-16.
[23] Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, et al. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis. 2009;30(3):377-86.
[24] Shirvani VN, Ouatu-Lascar R, Kaur BS, Omary MB, Triadafilopoulos G. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: Ex vivo induction by bile salts and acid exposure. Gastroenterology. 2000;118(3):487-96.
[25] Wilson KT, Fu S, Ramanujam KS, Meltzer SJ. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. Cancer Res. 1998;58(14):2929-34.
[26] Yu HP, Shi LY, Lu WH, Su YH, Li YY, Xu SQ. Expression of cyclooxygenase-2 (COX-2) in human esophageal cancer and in vitro inhibition by a specific COX-2 inhibitor, NS-398. J Gastroenterol Hepatol. 2004;19(6):638-42.
[27] Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K. Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res. 1999;59(1):198-204.
[28] Gupta RA, Tejada LV, Tong BJ, Das SK, Morrow JD, Dey SK, et al. Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer. Cancer Res. 2003;63(5):906-11.
[29] Daikoku T, Wang D, Tranguch S, Morrow JD, Orsulic S, DuBois RN, et al. Cyclooxygenase-1 is a potential target for prevention and treatment of ovarian epithelial cancer. Cancer Res. 2005;65(9):3735-44.
[30] Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science. 1994;265(5174):956-9.
[31] Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature. 1998;396(6706):77-80.
[32] Thoms HC, Dunlop MG, Stark LA. p38-mediated inactivation of cyclin D1/cyclin-dependent kinase 4 stimulates nucleolar translocation of RelA and apoptosis in colorectal cancer cells. Cancer Res. 2007;67(4):1660-9.
[33] Yu HG, Huang JA, Yang YN, Huang H, Luo HS, Yu JP, et al. The effects of acetylsalicylic acid on proliferation, apoptosis, and invasion of cyclooxygenase-2 negative colon cancer cells. Eur J Clin Invest. 2002;32(11):838-46.
[34] Din FV, Valanciute A, Houde VP, Zibrova D, Green KA, Sakamoto K, et al. Aspirin inhibits mTOR signaling, activates AMP-activated protein kinase, and induces autophagy in colorectal cancer cells. Gastroenterology. 2012;142(7):1504-15 e3.
[35] Hawley SA, Fullerton MD, Ross FA, Schertzer JD, Chevtzoff C, Walker KJ, et al. The ancient drug salicylate directly activates AMP-activated protein kinase. Science. 2012;336(6083):918-22.
[36] Liao X, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. N Engl J Med. 2012;367(17):1596-606.

[37] Deb J, Dibra H, Shan S, Rajan S, Manneh J, Kankipati CS, et al. Activity of aspirin analogues and vanillin in a human colorectal cancer cell line. Oncol Rep. 2011;26(3):557-65.
[38] Claudius AK, Kankipati CS, Kilari RS, Hassan S, Guest K, Russell ST, et al. Identification of aspirin analogues that repress NF-kappaB signalling and demonstrate anti-proliferative activity towards colorectal cancer in vitro and in vivo. Oncol Rep. 2014;32(4):1670-80.
[39] Zaugg RH, Walder JA, Walder RY, Steele JM, Klotz IM. Modification of hemoglobin with analogs of aspirin. J Biol Chem. 1980;255(7):2816-21.
[40] Rockett JC, Larkin K, Darnton SJ, Morris AG, Matthews HR. Five newly established oesophageal carcinoma cell lines: phenotypic and immunological characterization. Br J Cancer. 1997;75(2):258-63.
[41] Hughes SJ, Nambu Y, Soldes OS, Hamstra D, Rehemtulla A, Iannettoni MD, et al. Fas/APO-1 (CD95) is not translocated to the cell membrane in esophageal adenocarcinoma. Cancer Res. 1997;57(24):5571-8.
[42] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63.
[43] Chan M, Gravel M, Bramoulle A, Bridon G, Avizonis D, Shore GC, et al. Synergy between the NAMPT inhibitor GMX1777(8) and pemetrexed in non-small cell lung cancer cells is mediated by PARP activation and enhanced NAD consumption. Cancer Res. 2014;74(21):5948-54.
[44] Luo HY, Wei W, Shi YX, Chen XQ, Li YH, Wang F, et al. Cetuximab enhances the effect of oxaliplatin on hypoxic gastric cancer cell lines. Oncol Rep. 2010;23(6):1735-45.
[45] Toscano F, Parmentier B, Fajoui ZE, Estornes Y, Chayvialle JA, Saurin JC, et al. p53 dependent and independent sensitivity to oxaliplatin of colon cancer cells. Biochem Pharmacol. 2007;74(3):392-406.
[46] Yan KH, Yao CJ, Chang HY, Lai GM, Cheng AL, Chuang SE. The synergistic anticancer effect of troglitazone combined with aspirin causes cell cycle arrest and apoptosis in human lung cancer cells. Mol Carcinog. 2010;49(3):235-46.
[47] Zhou J, Zhou Y, Yin B, Hao W, Zhao L, Ju W, et al. 5-Fluorouracil and oxaliplatin modify the expression profiles of microRNAs in human colon cancer cells in vitro. Oncol Rep. 2010;23(1):121-8.
[48] Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984;22:27-55.
[49] Bijnsdorp IV, Giovannetti E, Peters GJ. Analysis of drug interactions. Methods Mol Biol. 2011;731:421-34.
[50] Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev. 2006;58(3):621-81.
[51] Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods. 1995;184(1):39-51.
[52] Alcindor T, Beauger N. Oxaliplatin: a review in the era of molecularly targeted therapy. Curr Oncol. 2011;18(1):18-25.
[53] Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010;70(2):440-6.
[54] Din FV, Dunlop MG, Stark LA. Evidence for colorectal cancer cell specificity of aspirin effects on NF kappa B signalling and apoptosis. Br J Cancer. 2004;91(2):381-8.
[55] Sundstrom J, Hedberg J, Thuresson M, Aarskog P, Johannesen KM, Oldgren J. Low-Dose Aspirin Discontinuation and Risk of Cardiovascular Events: A Swedish Nationwide, Population-Based Cohort Study. Circulation. 2017;136(13):1183-92.
[56] Dong H, Liu G, Jiang B, Guo J, Tao G, Yiu W, et al. The effects of aspirin plus cisplatin on SGC7901/CDDP cells in vitro. Biomed Rep. 2014;2(3):344-8.
[57] Shirakawa K, Wang L, Man N, Maksimoska J, Sorum AW, Lim HW, et al. Salicylate, diflunisal and their metabolites inhibit CBP/p300 and exhibit anticancer activity. Elife. 2016;5.

[58] Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY, Vilcek J. Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation. Proc Natl Acad Sci U S A. 1997;94(7):2869-73.